Exercise 1: Molecular Diagnostics

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Exercise 1

- Purpose
  - To test several different diagnostic methods:
    - FISH
    - 16S rRNA RFLP
    - Sequencing of 16s rRNA
    - Biochemical tests (BIOLOG and antibiotic sensitivity)
- Each team will identify 2 clinically isolates + one isolate from your body or the environment

Introduction

- Why identify bacteria:
  - Targeted treatment of infected patients
  - Test and control of food products
- How bacteria are identified:
  - Phenotypic
  - Genotypic

Phenotypic identification

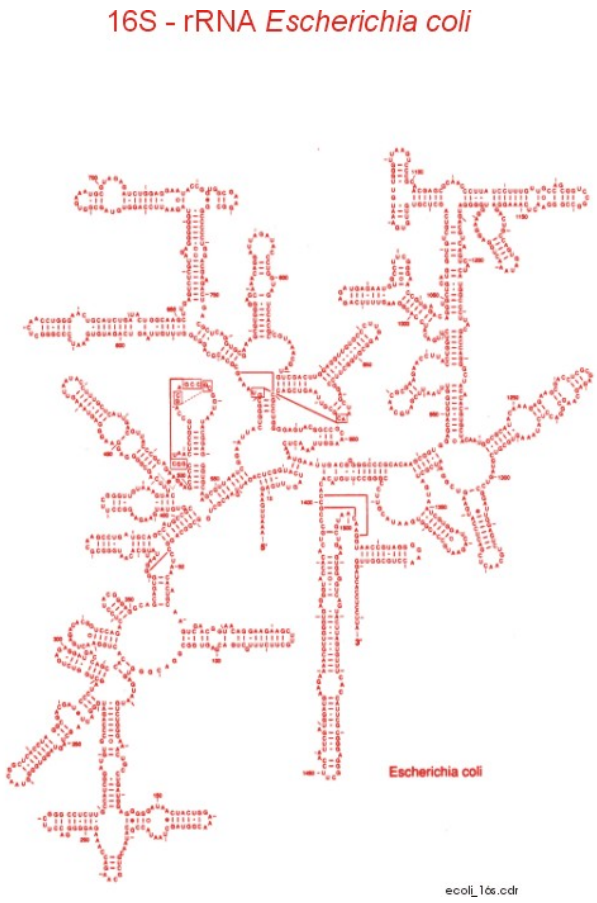
- Phenotypic identification:
  - Microscopy
  - Biochemical assays
    - testing for specific metabolisms or other traits
  - Staining
    - E. g. Gram staining

Genotypic identification

- Methods based on bacterial DNA/RNA
  - Fluorescent in situ hybridisation (FISH)
  - 16S rRNA restriction fragment length polymorphism (RFLP)
  - rRNA sequencing
- Advantages:
  - Fast, do not require cultivation, precise

FISH

- Based on 16S rRNA
- All bacteria have 16S rRNA
  - Conserved regions
  - Variable regions

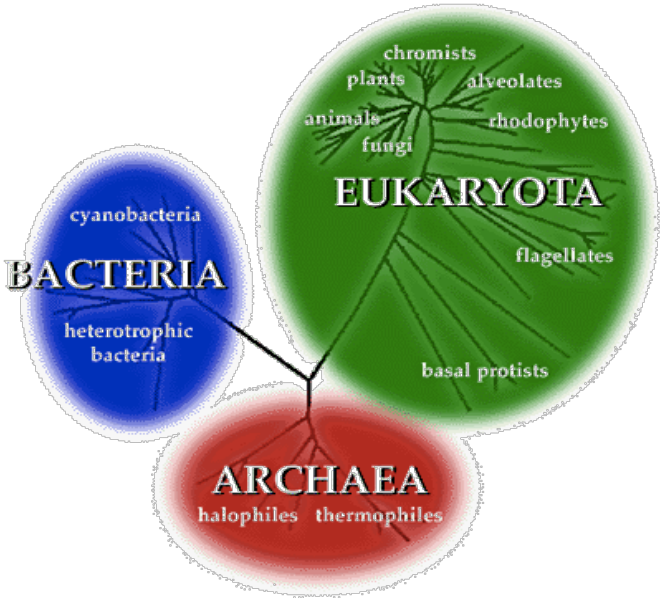


FISH

- Hybridisation of fluorescent probes complimentary to 16s rRNA

GATTCCTAGATTAGGTCGT

- Different levels:
  - Domain — Bacteria
  - Phylum — Proteobacteria
  - Class — Gamma Proteobacteria
  - Order — Pseudomonadales
  - Family — Pseudomonadaceae
  - Genus — *Pseudomonas*
  - Species — *aeruginosa*
  - Subspecies
  - Strain — PAO1



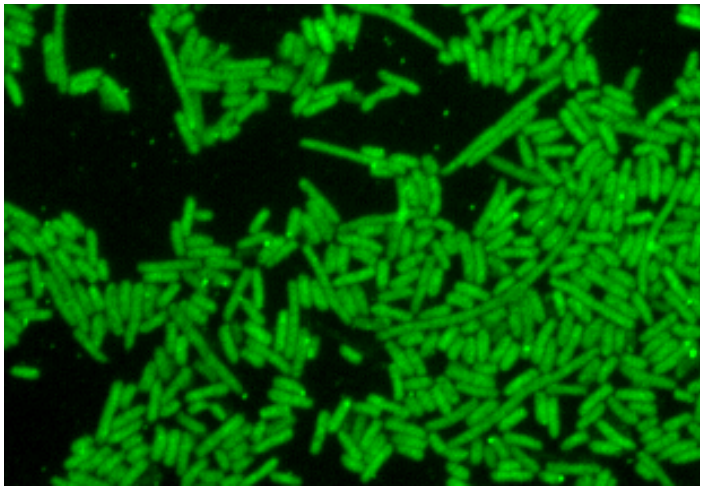
FISH

- Different levels of probes:
  - Domain
  - Phylum
  - Class
  - Order
  - Family
  - Genus
  - Species
  - Subspecies
  - Strain

Probe	Sequence 5' →3'	Label
β-proteobacteria	GCC TTC CCA CTT CGT TT	CY5 (Blue)
γ-proteobacteria	GCC TTC CCA CAT CGT TT	CY3 (Red)
Bacteria	GCT GCC TCC CGT AGG AGT	FITC (green)

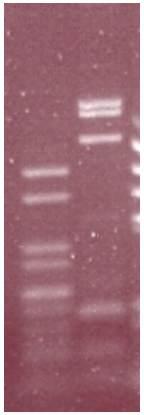
FISH

- Cells are fixed and made permeable
- Probes are hybridised
- The result is viewed using epifluorescence microscopy



16S rRNA RFLP

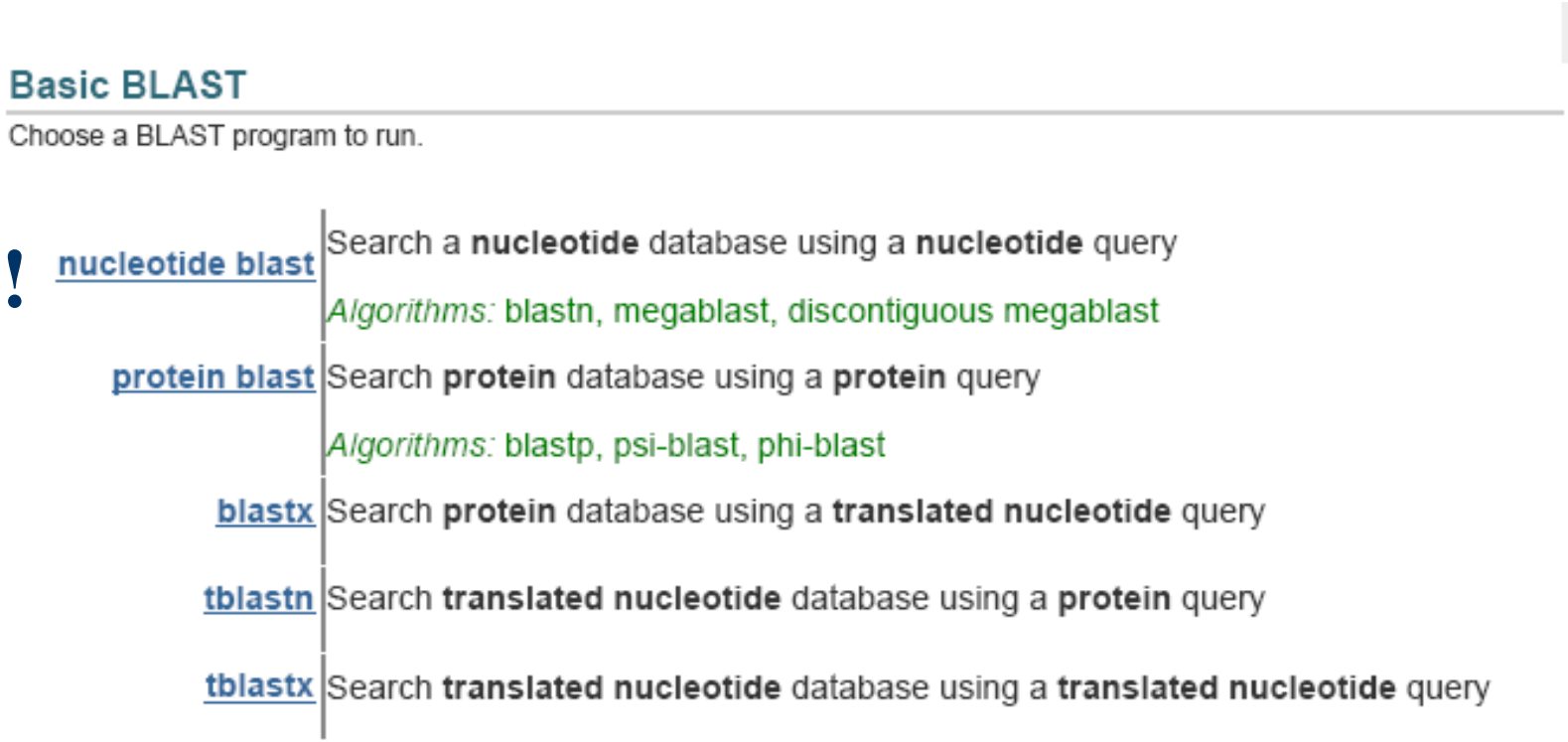
- Restriction Fragment Length Polymorphism
  - The gene encoding 16s rRNA is amplified by PCR
  - The PCR fragment is cut with restriction enzymes
  - Cut DNA is visualised using gel electrophoresis
  - Fragment pattern = fingerprint of the organism

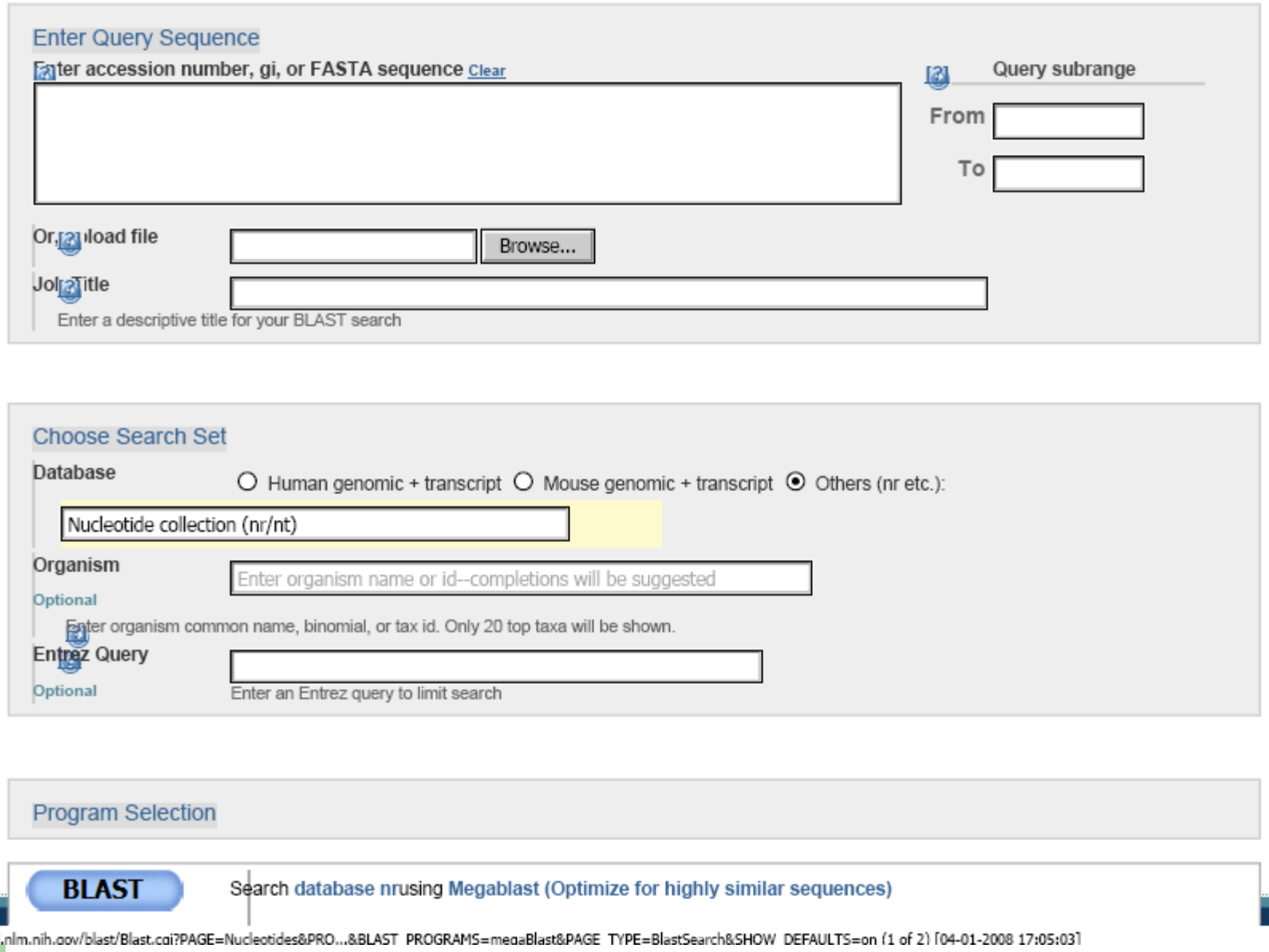


rDNA sequencing

- Using 16S rDNA sequencing, the strain or its relation can be determined specifically
- The sequencing usually takes place externally
- The sequence is compared with sequence databases on the internet
- Search tool: BLAST
- use: http://www.ncbi.nlm.nih.gov/

NCBI - Blast


Basic BLAST
Choose a BLAST program to run.
nucleotide blast
Search a nucleotide database using a nucleotide query
Algorithms: blastn, megablast, discontiguous megablast
protein blast
Search protein database using a protein query
Algorithms: blastp, psi-blast, phi-blast
blastx
Search protein database using a translated nucleotide query
tblastn
Search translated nucleotide database using a protein query
tblastx
Search translated nucleotide database using a translated nucleotide query


Enter Query Sequence
Enter accession number, gi, or FASTA sequence Clear
Query subrange
From
To
Or, upload file
Browse...
Job Title
Enter a descriptive title for your BLAST search
Choose Search Set
Database
Human genomic + transcript
Mouse genomic + transcript
Others (nr etc.):
Nucleotide collection (nr/nt)
Organism
Enter organism name or id--completions will be suggested
Optional
Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.
Entrez Query
Optional
Enter an Entrez query to limit search
Program Selection
BLAST
Search database nrusing Megablast (Optimize for highly similar sequences)

Blast

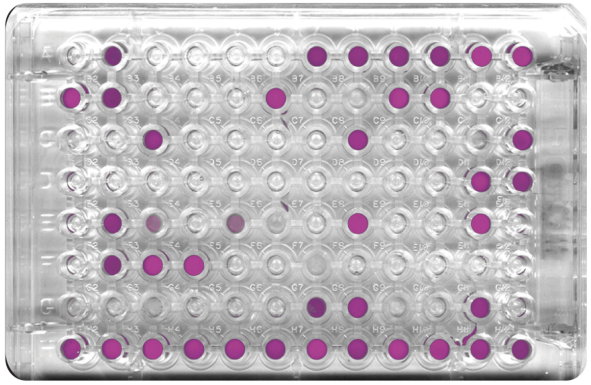
Sequences producing significant alignments:	Score (bits)	E Value
gi|41562|emb|X05173.1|ECGLN E.coli glnALG operon with glnA ...	952	0.0
gi|304961|gb|L19201.1|ECOUW87 E. coli chromosomal region fr...	952	0.0
gi|1790295|gb|AE000462.1|AE000462 Escherichia coli K12 MG16...	952	0.0
gi|13364198|dbj|AP002567.1| Escherichia coli O157:H7 DNA, c...	928	0.0
gi|12518741|gb|AE005617.1|AE005617 Escherichia coli O157:H7...	928	0.0
gi|146156|gb|M13746.1|ECOGLNAB E.coli glnA gene encoding gl...	920	0.0
gi|24054430|gb|AE015401.1| Shigella flexneri 2a str. 301 se...	912	0.0
gi|30042943|gb|AE016990.1| Shigella flexneri 2a str. 2457T ...	912	0.0
gi|26111017|gb|AE016770.1| Escherichia coli CFT073 section ...	864	0.0
gi|16422561|gb|AE008887.1| Salmonella typhimurium LT2, sect...	500	e-138
gi|154089|gb|M14536.1|STYGLNA S.typhimurium glnA gene encod...	500	e-138
gi|29139451|gb|AE016846.1| Salmonella enterica subsp. enter...	492	e-136
gi|16504729|emb|AL627280.1| Salmonella enterica serovar Typ...	492	e-136
gi|3808289|gb|AF072440.1|AF072440 Enterobacter gergoviae GT...	468	e-129
gi|23630224|gb|AY144623.1| Pantoea agglomerans glutamine sy...	389	e-105
gi|21960789|gb|AE013983.1| Yersinia pestis KIM section 383 ...	309	3e-81
gi|15978115|emb|AJ414141.1| Yersinia pestis strain CO92 com...	309	3e-81
gi|45434720|gb|AE017127.1| Yersinia pestis biovar Mediaevai...	309	3e-81
gi|146158|gb|J01618.1|ECOGLNACR Escherichia coli strain K-1...	256	4e-65
gi|33087906|gb|AY333022.1| Yersinia bercovieri strain WS 39...	230	2e-57
gi|33087902|gb|AY333020.1| Yersinia bercovieri strain WS 19...	226	3e-56
gi|33087898|gb|AY333018.1| Yersinia bercovieri strain WA 17...	226	3e-56
gi|33087896|gb|AY333017.1| Yersinia bercovieri strain ATCC ...	226	3e-56
gi|33087952|gb|AY333045.1| Yersinia kristensenii strain WA ...	202	5e-49
gi|33087916|gb|AY333027.1| Yersinia enterocolitica strain 1...	202	5e-49

Biochemical Tests

- BIOLOG test
  - Based on the ability of the bacteria to utilize different carbon sources
- Antibiotic sensitivity
  - Important for treatment and prognosis
  - Otherwise ”identical” strains can differ in AB pattern

Biolog GEN III system

96 wells microplate assay containing different C-sources and antibiotics



Growth pattern identify the bacteria down to species level

GEN III MicroPlate ™

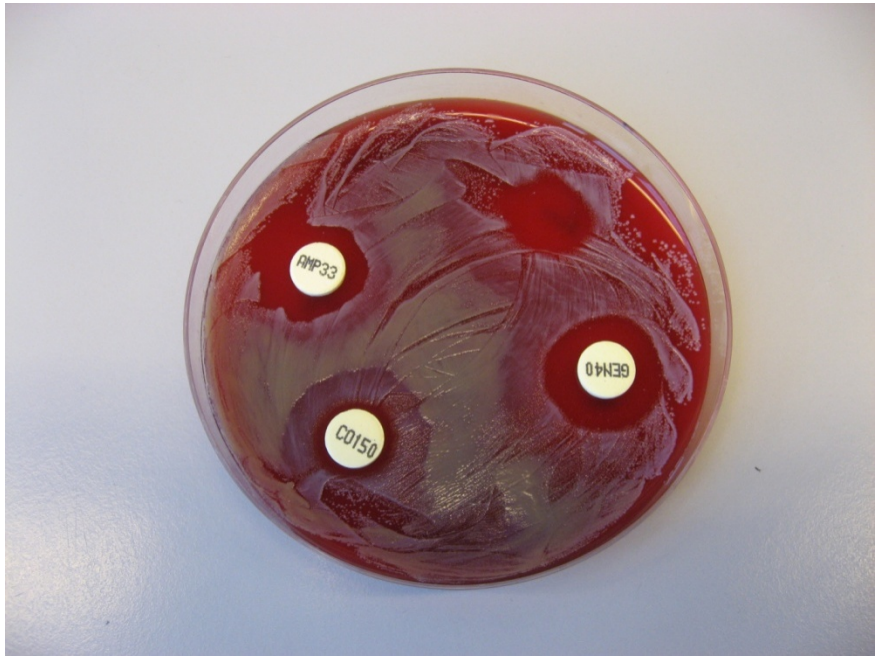
A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Antibiotic Testing

There are several ways of determining the sensibility to different antibiotics.

They will be addressed in Ex 2.

In Ex 1 we use ”Rosco- tabs”



Exercise 1

- Remember:
  - Plan the experiments
  - Use GLOVES
  - Work sterile
  - All cell-containing liquids, supernatants etc. must be disposed in the culture disposal flasks in the fume hood.

- Fixation and hybridisation of cells: MUST be in the FUME HOOD

Exercise 1. Flow chart

Day	1 A FISH	1 B PCR	1 C RFLP	1 D Antibiotic test	1 E BIOLOG ID
Monday 10/1	Inoculate liquid media (also used in 1B)			Inoculate plate media (BA). Sample own isolate.	
Tuesday 11/1	Fix cells (do this first today). FISH Procedure.	Prepare Chromosomal DNA			
Wednesday 12/1	Microscopy of FISH samples. Can be continued Thursday	16S PCR amplification. (also used in 1C)		AB test plate preparation.	
Thursday 13/1			Restriction enzyme cut PCR products	AB and Blue plate inoculation	Inoculate BIOLOG plates
Friday 14/1			Run agarose gel Take Gel photo	Read Plates	Read BIOLOG plates

Preparation of Chromosomal DNA

1. Harvest 500 µl culture : Spin down cells (5 min at 7000 g) and decant.
2. Wash with 1 ml TNE : Resuspend in 1 ml TNE; vortex; spin down cells (5 min at 7000 g); decant.
3. Resuspend pellet in 270 µl TNEX.
4. Add 30 µl of a freshly prepared lysozyme solution (5 mg/ml in H_2O).
5. Add 7.5 µl Proteinase K solution (20 mg/ml in H_2O).
6. Incubate for 90 min at 37°C and then at 65°C for another 90 min.
7. Add 15 µl 5 M NaCl and mix gently by inverting the tube.
8. Add gently 1 ml 96% EtOH. After 2-3 min mix gently and incubate 15 min at -20°C.
9. Spin at 15000 g for 10 min at 4°C. → DNA visible
10. Wash pellet with 1 ml ice cold 96% EtOH. Be careful! Don't loose the DNA!
11. Spin at 15000 g for 10 min at 4°C.
12. Remove remaining EtOH by pipetting followed by incubation at 37°C for 3-5 min with the lid open (do not over dry pellet).
13. Add 500 µl dH_2O and mix gently by inverting the tube. Store at 20°C o.n. to dissolve the DNA; then at -20°C.

Don't vortex chromosomal DNA or it will shear.

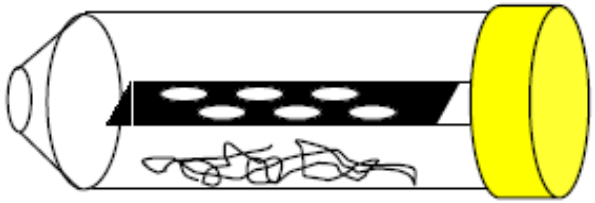
FISH day 2

- 2 Fume hoods + 1 incubator chamber 37 degree

- Fixation of bacteria:
  - Fixative waste in the container
  - Use blue (nitril) gloves
  - Recommended to continue right away with the hybridization step

FISH day 2

- Hybridization step:
  - Probes are ready for use
  - 3 probes separately, so three wells per strain!
  - One 37 degree incubator for the 3h hybridization



  - Slides are collected when finished (remember name)

FISH day 3

- Visualization of hybridized cells using epifluorescence microscopy (1. floor, room 125)
- Time schedule starting from 9:30 and running for the rest of the day
- New team roughly every 30-45 minutes

Team 1: 9:30
Team 2: 10:15
Team 3: 11:00
Team 4: 11:45
Team 5: 13:00
Team 6: 13:45
Team 7: 14:30
Team 8: 15:00
Team 9: 15:45
Team 10: 16:30